METHOD FOR GENERATING FULL-LENGTH MESSENGER RNA LIBRARY

CROSS REFERENCE OF RELATED APPLICATION

This is a regular application of a provisional application, application number 60/125,695, filed 03/23/1999.

BACKGROUND OF THE INVENTION

1. Field of The Invention

The present invention generally relates to the field of methods for generating amplified messenger RNA sequences. More particularly, the present invention relates to the field of multiple polymerase reaction methods of full-length messenger RNA library amplification from single cells.

2. Description of The Prior Art

The following references are pertinent to this invention:

1. Sambrook et.al., "*Molecular Cloning, 2nd Edition* ",Cold Spring Harbor Laboratory Press, pp8.11-8.19 (1989).
2. Van Gelder et.al., "Amplified RNA synthesized from limited quantities of heterogeneous cDNA", *Proc. Natl. Acad. Sci. USA* 87: 1663-1667 (1990).

3. O'Dell et.al., "Amplification of mRNAs from Single, Fixed, TUNEL-Positive Cells", *BioTechniques* 25: 566-570 (1998).

4. Eberwine et.al. , "Analysis of gene expression in single live neurons", *Proc. Natl. Acad. Sci. USA* 89: 3010-3014 (1992).

5. Shi-Lung Lin, Cheng-Ming Chuong, Randall B. Widelitz and Shao-Yao Ying; In Vivo Analysis of Cancerous Gene Expression by RNA-Polymerase Chain Reaction. *Nucleic Acid Res.* 27: 4585-4589 (1999).

6. Embleton et.al., "In-cell PCR from mRNA: amplifying and linking the rearranged immunoglobulin heavy and light chain V-genes within single cells", *Nucleic Acid Res.* 20: 3831-3837 (1992).

7. United States Patent No. 4,683,202 issued to Mullis et.al.

8. United States Patent No. 5,817,465 issued to Mallet et.al.

9. United States Patent No. 5,514,545 issued to Eberwine et.al.

The ability to amplify messenger RNAs (mRNA) directly from single cells has permitted the molecular investigations of intracellular gene expressions under certain special conditions, such as pathogenesis, mutation, treatment processing and developmental control. In general, mRNAs are reverse-transcribed to complementary DNAs (cDNA) for preventing degradation. Because the integrity and quantity of mRNAs determine the completeness of a full-length cDNA library, the acquirement of large amount of high quality mRNAs from single cells is the major problem in the sample preparations of various genetic research. Especially, when mRNAs from single cells of a special tissue are used to generate a cDNA library, the library must contain intact gene expression pattern in order to identify tissue-specific genes differentially expressed in vivo. Although previous methods

for cDNA library synthesis (Sambrook et.al., "*Molecular Cloning, 2nd Edition* ", pp8.11-8.35 (1989)) have succeeded in generating full-length cDNAs to represent related mRNAs, the tedious procedures of reverse transcription, restriction, adaptor ligation and vector cloning usually fail to maintain the completeness of representative cDNA sequences, resulting in a loss of rare mRNA representatives when limited cells are used.

On the other hand, the amplification of mRNA-representative cDNAs by reverse transcription-polymerase chain reaction (RT-PCR) has become the most common way among current RNA representative amplification methods. Prior art attempts at amplifying mRNA-representatives with RT-PCR, such as United States Patent No. 4,683,202 to Mullis and United States Patent No. 5,817,465 to Mallet, uses mRNA templates to multiply respective cDNAs. Although the RT-PCR methods successfully increase the quantity of double-stranded cDNAs from their respective RNAs, the fidelity of resulting cDNAs is not completely identical to their RNA origins due to the mis-reading nature of polymerase chain reaction (PCR). Moreover, the final products of RT-PCR are double-stranded DNAs, rather than real mRNAs, which can not be used in probe hybridization and in vitro translation. Furthermore, current RT-PCR methods are not designed for the amplification of full-length mRNAs or cDNAs, indicating the impossibility of generating a full-length cDNA library by this kind of methods. Those disadvantages exclude the use of RT-PCR products in the analysis of unknown gene expressions.

The generation of amplified antisense RNAs (aRNA) has been developed to increase transcriptional copies of specific mRNAs from single cells (Van Gelder et.al., *Proc. Natl. Acad. Sci. USA* 87: 1663-1667 (1990)). The aRNA can be used for characterization of the intracellular expression pattern of certain genes, but not all gene repertoires (O'Dell et.al., *BioTechniques* 25: 566-570 (1998)). Prior art attempts at aRNA amplification, such as United States Patent No. 5,514,545 to Eberwine, uses the amplified aRNAs as probes for potential gene diagnosis and therapy. By incorporating a poly(dT)

primer coupled to a T7 RNA polymerase promoter sequence, named oligo(dT)-promoter primer, during reverse transcription (RT), partial mRNA sequences can be amplified up to two thousand folds in the form of antisense conformation which is complementary to the natural mRNAs (Eberwine et.al. , *Proc. Natl. Acad. Sci. USA* 89: 3010-3014 (1992)). Although these aRNA amplification methods lead to the identification of some abundant mRNA representative markers from single cells, the rare mRNA representatives can not be assessable by the current aRNA methods (O'Dell et.al., *BioTechniques* (1998)), resulting in low completeness of aRNA libraries. Moreover, the aRNA products are partial "antisense" RNAs rather than natural full-length "messenger" RNAs which are in the "sense" conformation. In fact, we can use the full-length mRNAs to synthesize functional proteins in vitro, but not the aRNAs.

In summary, it is desirable to have a fast, simple and specific method for generating amplifiable full-length mRNAs rather than aRNAs from single cells, of which the results may be applied to screen differentially expressed genes, to search functional domains for gene regulation, to produce synthetic peptides in vitro, and even to design a therapy for diseases.

SUMMARY OF THE INVENTION

The present invention is a novel polymerase chain reaction method which amplifies messenger RNAs from single cells.

Described in detail, a preferred embodiment of the present invention method includes the following steps:

(a) preventing a plurality of messenger RNAs from degradation, wherein said messenger RNAs are protected to be intact along with following steps;

(b) contacting said messenger RNAs with a plurality of oligodeoxythymidylate nucleotide sequences to form a plurality of first-strand complementary DNAs, wherein said first-strand complementary DNAs are generated by reverse transcription of said messenger RNAs with said oligodeoxythymidylate nucleotide sequences as primers;

(c) permitting 3'-end extension of said first-strand complementary DNAs to form a plurality of polynucleotide-tailed first-strand complementary DNAs, wherein said polynucleotide-tailed first-strand complementary DNAs are extended by terminal transferase activity with multiple copies of same deoxynucleotides to form polynucleotide tails;

(d) incubating denatured said polynucleotide-tailed first-strand complementary DNAs with a plurality of oligo(antisense polynucleotide)-promoter primers to form a plurality of double-stranded complementary DNAs, wherein said double-stranded complementary DNAs are generated by extension of DNA polymerase activity with said oligo(antisense polynucleotide)-promoter primers complementary to the polynucleotide tails of said polynucleotide-tailed first-strand complementary DNAs;

(e) permitting transcription of said double-stranded complementary DNAs to form a plurality of amplified messenger RNAs, wherein said amplified messenger RNAs are generated by extension of RNA polymerase activity through the promoter region of said double-stranded complementary DNAs; and

(f) contacting said amplified messenger RNAs with said oligodeoxythymidylate nucleotide sequences to form a plurality of said polynucleotide-tailed first-strand complementary DNAs, wherein said polynucleotide-tailed first-strand complementary DNAs are generated by reverse transcription of said amplified messenger RNAs with said oligodeoxythymidylate nucleotide sequences as primer.

In one aspect of this embodiment, the cycling steps of (d) through (f) can be repeated at least one time for the amplification of said messenger RNAs. According to another aspect of this preferred embodiment, the final nucleotide products are preserved in the form of double-stranded duplexes to prevent the degradation of amplified messenger RNAs, preferably, in the form of RNA-DNA hybrid duplexes in the step (f).

The mRNAs can be prepared from a plurality of fixed cells, wherein said fixed cells are protected from RNA degradation and also subjected to permeabilisation for enzyme penetration. Those fixed cells include fixative-treated cultural cells, frozen fresh tissues, fixative-treated fresh tissues or paraffin-embedded tissues on slides. To increase the transcriptional production of mRNAs in the step (e), the promoter sequences are preferably incorporated into the 5'-ends of said second-strand cDNAs. In another aspect of this embodiment, said amplified mRNAs are preferably capped by P^1-5'-(7-methyl)-guanosine-P^3-5'-adenosine-triphosphate or P^1-5'-(7-methyl)-guanosine-P^3-5'-guanosine-triphosphate in the step (e) for further in vitro translation. On the other hand, the deoxynucleotide used in the tailing reaction of said first-strand complementary DNAs is either deoxyguanylate (dG) or deoxycytidylate (dC), and the average number of tailed nucleotides is larger than seven; most preferably, the number is about twelve. Advantageously, the final amplified mRNAs can be continuously reverse-transcribed into double-stranded cDNA by Tth-like DNA polymerase activity. The final double-stranded cDNAs are preferably cloned into

competent vectors for further applications, such as transfection assay, differential screening, functional detection and so on.

Further novel features and other objects of the present invention will become apparent from the following detailed description, discussion and the appended claims, taken in conjunction with the drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Referring particularly to the drawings for the purpose of illustration only and not limitation, there is illustrated:

FIG.1 is an illustration of the preferred embodiment of RNA-polymerase chain reaction of the subject invention;

FIG.2 is an illustration of second preferred embodiment of RNA-polymerase chain reaction of the subject invention;

FIG.3 is an illustration of third preferred embodiment of the RNA-polymerase chain reaction of the subject invention; and

FIGS.4a and 4b are the results of example 4 of the subject invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Although specific embodiments of the present invention will now be described with reference to the drawings, it should be understood that such embodiments are by way of

example only and merely illustrative of but a small number of the many possible specific embodiments which can represent applications of the principles of the present invention. Various changes and modifications obvious to one skilled in the art to which the present invention pertains are deemed to be within the spirit, scope and contemplation of the present invention as further defined in the appended claims.

The present invention is directed to a novel polymerase chain reaction method for mRNA amplification from single cells, named "RNA-polymerase chain reaction (RNA-PCR)". This method is primarily designed for differential screening of tissue-specific gene expressions in cell level, cloning full-length sequences of unknown gene transcripts, generating pure probes for hybridization assays, synthesizing peptides in vitro, and preparing complete cDNA libraries for gene chip technology. The purpose of the RNA-PCR relies on the repeating steps of reverse transcription, denaturation, double-stranded cDNA synthesis and in vitro transcription to bring up the population of mRNAs to two thousand folds in one cycle of above procedure. In brief, the preferred version (FIG.1) of the present invention is based on: 1) prevention of mRNA degradation, 2) first reverse transcription and terminal transferase reaction to incorporate 3'-polynucleotide tails to the first-strand cDNAs, 3) denaturation and then double-stranded cDNA formation based on the extension of specific promoter-primers complementary to the 3'-polynucleotide tails, 4) transcription from promoter to amplify mRNAs up to two thousand folds per round, and 5) repeating aforementioned steps to achieve desired RNA amplification.

Alternatively, the second preferred version (FIG.2) of the present invention is based on: 1) prevention of mRNA degradation, 2) first reverse transcription to incorporate first promoters to the 5'-ends of first-strand cDNAs and then addition of polynucleotide sequences to the 3'-ends of the first-strand cDNAs, 3) double-stranded cDNA synthesis based on the extension of second promoter sequences complementary to the 3'-polynucleotide regions of the first-strand cDNAs, 4) transcription to amplify either aRNAs

or mRNAs up to two thousand folds in the first round of amplification cycle, and 5) repeating aforementioned cycling steps to achieve desired amount of RNAs. As shown in FIG.2, the first promoter used here is different from the second promoter, resulting the control of transcription by adding different RNA polymerases. The first promoter is incorporated for aRNA amplification, whereas the second promoter is designed for mRNA amplification. By this way in conjunction with a reverse transcription step, we can choose to amplify aRNAs, first-strand cDNAs, mRNAs or second-strand cDNAs of interest, depending on which RNA polymerase and nuclease we use. Although the second and third preferred embodiments (FIGS.2 and 3) are more complicated than the first preferred embodiment (FIG.1), the principle and broad features of the second and third preferred embodiments are completely within the scope of the first preferred embodiment of the present invention.

As used herein, the first-strand complementary DNA (cDNA) refers to a DNA sequence which is complementary to a natural messenger RNA sequence in an A-T and C-G composition. The antisense RNA (aRNA) refers to an RNA sequence which is complementary to a natural messenger RNA sequence in an A-U and C-G composition. And, the oligo(dT)-promoter sequence refers to an RNA polymerase promoter sequence coupled with a poly-deoxythymidylate (dT) sequence in its 3'-end, of which the minimal number of linked dT is seven; most preferably, the number is about twenty-six. The sense sequence refers to a nucleotide sequence which is in the same sequence order and composition as its homolog mRNA, whereas the antisense sequence refers to a nucleotide sequence which is complementary to its respective mRNA homologue. On the other hand, the oligo(antisense polynucleotide)-promoter sequence refers to an oligonucleotide sequence which is complementary to the polynucleotide-tail of said polynucleotide-tailed cDNAs and also linked to an RNA polymerase promoter in its 5'-end. And, Tth-like DNA polymerases refer to RNA- and DNA-dependent DNA polymerases with reverse transcription activity.

By improving the methods of in-vitro transcription and in-cell RT-PCR (Embleton et.al., *Nucleic Acid Res.* (1992)), we invent the cycling amplification of intracellular full-length mRNAs. This cycling procedure preferably starts from reverse transcription of intracellular mRNAs with Tth-like DNA polymerase, following a tailing reaction with terminal transferases and then denaturation of resulting mRNA-cDNA hybrid duplexes. After renaturation of above tailed cDNAs to specific promoter-linked primers, double-stranded cDNAs are formed by Tth-like DNA polymerases. And then, promoter-specific RNA polymerase(s) is added to accomplish the transcriptional amplification of intracellular mRNAs. The novelties of this amplification cycling procedure of the present invention are as follows: 1) single copy/rare mRNAs can be increased up to 2000 folds in one round of amplification without mis-reading mistakes, 2) the mRNA amplification is linear and does not result in preferential amplification of abundant mRNA species, 3) the mRNA degradation is inhibited by fixation, and 4) the final mRNA products are of full-length and can be directly used to generate a complete cDNA library or synthesize proteins in vitro (Shi-Lung Lin et.al. *Nucleic Acid Res.* (1999)).

In the second preferred embodiment, referring to FIG.2, when the promoter of oligo(dT)-promoter primers is different from that of oligo(antisense polynucleotide)-promoter primers, the amplification of aRNAs and mRNAs can be separated by adding different RNA polymerases in the step (e), but not both. However, in the third preferred embodiment, referring to FIG. 3, when the promoter of oligo(dT)-promoter primers is the same as that of oligo(antisense polynucleotide)-promoter primers, the amplification of aRNAs and mRNAs must be separated by adding same RNA polymerase in the different steps, such as (e) and (h). Both preferred embodiments are capable of fulfill the purpose of the present invention to amplify mRNAs from single cells. Although we currently need to add new RNA polymerase in every round of transcription due to the denaturation step, the finding of thermostable RNA polymerases may make the procedure of the present invention more convenient. For example, if thermostable RNA polymerases become available, the

amplification cycle can be directly completed in a microtube by following the first preferred embodiment (FIG.1) of the present invention. Examples as mentioned here will be developed into continuation in part of the present invention and is not intended in any way to limit the broad features or principles of the present invention.

In stead of poly(dT), oligo(dT)-promoter, antisense polynucleotide and oligo(antisense polynucleotide)-promoter primers, we can use sequence-specific primers and sequence-specific promoter-linked primers to accomplish the amplification of normalized aRNAs, mRNAs, first-strand cDNAs and second-strand cDNAs of interest. The labeling of cDNAs is accomplished by incorporation of labeled nucleotides or analogs during reverse transcription of Tth-like DNA polymerase activity, while that of the RNAs is completed during transcription . The nucleotide sequences so generated are capable of being probes in a variety of applications, such as Northern blots, Southern blots, dot hybridization, in situ hybridization, position cloning, antisense knockout transfection and so on. Alternatively, the preferred embodiments (FIGS.1, 2 and 3) provide amplified full-length mRNAs for in vitro translation. A cap-nucleotide can be added to the 5'-end of amplified mRNAs during the transcription step of the present invention. Unlike normalized RNAs, the capped mRNAs can be directly used in protein synthesis and may help the isolation of such protein. The preferred cap-nucleotides include P^1-5'-(7-methyl)-guanosine-P^3-5'-adenosine-triphosphate and P^1-5'-(7-methyl)-guanosine-P^3-5'-guanosine-triphosphate.

On the other hand, the first step of the present invention can start from fixed cells as well as mRNAs; i.e., fixed cultured cells, frozen fresh tissues, fixed tissues or tissues in slides. Since this formed mRNAs are of full-length and carry RNA promoter regions for in vitro/vivo expression, the transfection of certain gene transcript can be directly performed after its respective double-stranded cDNA is cloned into a competent vector. On the other hand, the present invention are also very useful in preparing complete full-length cDNA

libraries for modern gene chip technology. Because the present invention is capable of generating a complete repertoire of full-length cDNAs from single cells, tissue-specific cDNA libraries based on special cell types can be formed and transferred onto a filter, membrane or chip for preserving these genetic information. As we all have different genetic information from a variety of major tissues and organs, the cDNA-encoded gene chips may function as an individual source for differential screening, pathological diagnosis, physiological prognosis and genetic identification. This kind of approach will become more and more important following the completion of human genome project in the year 2003.

In the preferred embodiments (as shown in FIG.1, 2 and 3) of the present invention, according to the high amplification rate of RNA polymerase (about 2000 folds/cycle), the labor- and time-consuming factors in this RNA-PCR can be reduced to the minimum. Also, the preparation of amplified mRNAs is cheaper and more efficient than poly(dT)-linked chromatography columns in previous methods. Most importantly, this RNA amplification can be carried out in microtubes with only few cells. Taken together, these special features make the content of RNA-PCR as simple, fast, and inexpensive as a kit for concisely isolating amplified mRNA sequences of interest.

Although certain preferred embodiments of the present invention have been described, the spirit and scope of the invention is by no means restricted to what is described above. For example, within the general framework of: a) one or more specific primers for reverse transcription and polymerase extension reaction; b) one or more RNA promoter-primers for transcription; c) seven or more deoxynucleotides extended in a promoter-linked polynucleotide primer; d) seven or more same nucleotides added to the 3'-end of the first-strand cDNAs ; e) one or more rounds of the cycling steps for RNA amplification, there is a very large number of permutations and combinations possible, all of which are within the scope of the present invention.

EXAMPLE 1

Cell Fixation and Permeabilisation

LNCaP cells, a prostate cancer cell line, were grown in RPMI 1640 medium supplemented with 2% fetal calf serum. One 70% full of cells cultured in 60mm dish were trypsinized, collected and washed three times in 5ml phosphate buffered saline (PBS, pH 7.2) at room temperature, then suspended in 1ml of ice-cold 10% formaldehyde solution in 0.15M NaCl. After one hour incubation on ice with occasional agitation, the cells were centrifuged at 13,000rpm for 2 min and wash three times in ice-cold PBS with vigorous pipetting. The collected cells were resuspended in 0.5% Nonidet P40 (NP40, B.D.H.) and incubated for one hour with frequent agitation. After that, three washes were given to cells in ice-cold PBS containing 0.1M glycine and the cells were resuspended in 1ml of the same buffer with vigorous pipetting in order to be evenly separated into small aliquots and stored at -70^{0}C for up to a month.

EXAMPLE 2

First Reverse Transcription and Polynucleotide Tailing of The First-Strand cDNAs

For first reverse transcription of mRNAs in cells, one hundred of the fixed cells were thawed, resuspended in 20μl of DEPC-treated ddH_2O, mixed with 25pmol oligo(dT)-T7 promoter (SEQ ID.1), heated to 65^{0}C for 5 min and then cooled on ice. A 50μl RT reaction was prepared, comprising 10μl of 5x Mg-containing RT buffer (Boehringer Mannheim), dNTPs (1mM each for dATP, dGTP, dCTP and dTTP), RNase inhibitor and above cooled cells. After C. therm. polymerase (5U) was added, the RT reaction was

mixed and incubated at 55^{0}C for 10 min and shifted to 72^{0}C for one hour, and then the cells were washed once with PBS and resuspended in a 50μl tailing reaction, comprising 2mM dGTP, 10μl of 5x tailing buffer (250mM KCl, 50mM Tris-HCl, 8mM $MgCl_2$, pH 8.3 at 20^{0}C). The tailing reaction was heated at 94^{0}C for 3 min and then chilled in ice for mixing with terminal transferase (20U), following further incubation at 37^{0}C for 20 min. This formed said polynucleotide-tailed first-strand cDNAs.

EXAMPLE 3

Denaturation, Double-Stranded cDNA Synthesis and Transcriptional Amplification

Above tailing reaction was ~~stooped~~ stopped at 94^{0}C for 2 min, mixed with 25pmol poly(dC) primer (SEQ ID.2), and denatured at 94^{0}C for 3 more min. After 1 min centrifuging at room temperature, 1mM dNTPs and C. therm. polymerase (5U) were added to form double-stranded cDNAs at 70^{0}C for 5 min. To increase the amount of desired RNAs, the T7 promoter-linked first-strand cDNA was served as a coding strand for the transcription of T7 RNA polymerase (Eberwine et.al. (1992)). As few as several cells in 5μl of above resulting reaction can be used to accomplish full-length aRNA amplification during following reaction. A transcription reaction (50μl) was prepared, containing 5μl of 10x transcription buffer (Boehringer Mannheim), rNTPs (2mM each for ATP, GTP, CTP and UTP), RNA inhibitor and T7 RNA polymerase (2000U). After three hour incubation at 37^{0}C, the cDNA transcripts were isolated from both cells and supernatant, and can be directly used in following reverse transcription. The reaction was finally stopped at 94^{0}C for 3 min and chilled in ice immediately.

EXAMPLE 4

Second Reverse Transcription, Denaturation, Double-Stranded cDNA Synthesis and mRNA Amplification

A 50μl RT reaction was prepared, comprising 10μl of 5x Mg-containing RT buffer (Boehringer Mannheim), 25pmol oligo(dC)-SP6 promoter primer (SEQ ID.3), 2mM dNTPs, RNase inhibitor and 5μl of above aRNA-containing supernatant. After C. therm. polymerase (5U) was added, the RT reaction was mixed and incubated at 55^{0}C for 10 min and shifted to 72^{0}C for one hour. This formed said second-strand cDNAs. After another denaturation at 94^{0}C for 3 min and mixing with 25pmol poly(dT) primer (SEQ ID.4) at room temperature for 1 min, double-stranded cDNAs can be formed by adding 1mM dNTPs at 70^{0}C for 5 min. A transcription reaction (50μl) was then prepared to generate said amplified mRNAs, containing 5μl of 10x transcription buffer (Boehringer Mannheim), 2mM rNTPs, RNA inhibitor and SP6 RNA polymerase (2000U). After three hour incubation at 37^{0}C, the cDNA transcripts were isolated and can be directly used in another round of RNA-PCR. The final reaction was stopped at 94^{0}C for 3 min and chilled in ice immediately.. The quality of final amplified mRNAs (2μg) was assessed on a 1% formaldehyde-agarose gel, ranging from 500 bp to above 10kb (FIG.4a). We also have successfully identified the gene transcripts of RB, p21, β-actin and GAPDH on the Northern blots of full-ranged LNCaP mRNAs made by the present invention (FIG.4b).

EXAMPLE 5

Amplification Cycling Procedure

Few fixed cells were applied to a reverse transcription reaction (50μl) on ice, comprising 10μl of 5x RT&T buffer (100mM Tris-HCl, pH8.3 at 25^{0}C, 600mM KCl, 300mM $(NH_4)_2SO_4$, 40mM $MgCl_2$, 5M betaine, 35mM DTE and 10mM spermidine), 1μM poly(dT) primer (SEQ ID.4), dNTPs and RNase inhibitors (10U). After C. therm. polymerase (6U) was added, the reaction was incubated at 52^{0}C for 3min and shifted to 65^{0}C for another 30min. The first-strand cDNAs so obtained were collected in a tailing reaction (50μl), comprising 10μl of 5x tailing buffer (250mM KCl, 100mM Tris-HCl, 4mM $CoCl_2$, 10mM $MgCl_2$, pH8.3 at 20^{0}C) and 0.5mM dGTP. After terminal transferase (75U) was added, the reaction was incubated at 37^{0}C for 15min, stopped by denaturation at 94^{0}C for 2min and mixed with 1μM oligo$(dC)_{10}$-T7 promoter primer (SEQ ID.5). Taq DNA polymerase (3.5U) and dNTPs were then added to double-stranding above tailed cDNAs at 52^{0}C for 3min and then 72^{0}C for 7min. A transcription reaction (50μl) was prepared, containing 10μl of 5x RT&T buffer, 2mM rNTPs, RNA inhibitors (10U), T7 RNA polymerase (200U) and the promoter-linked double-stranded cDNAs. After one hours incubation at 37^{0}C, the mRNA transcripts were isolated and used directly for the next round of RNA-PCR without the tailing reaction, containing 10μl of 5x RT&T buffer, 1 μM poly(dT) primers, 1 μM oligo$(dC)_{10}$-promoter primers, 2mM dNTPs, 2mM rNTPs, C. therm. polymerase, Taq DNA polymerase and the mRNA products. The quality of mRNA products can be assessed on a 1% formaldehyde-agarose gel (Shi-Lung Lin et.al. *Nucleic Acid Res.* (1999)).

The present invention has been described with reference to particular preferred embodiments; however, the scope of this invention is defined by the attached claims and should be constructed to include reasonable equivalents.

Defined in detail, the present invention is a polymerase chain reaction method of generating amplified messenger RNAs from single cells, comprising the steps of:

a. providing a plurality of messenger RNAs for following steps;

b. contacting said messenger RNAs with a plurality of first primer sequences to form a plurality of first-strand complementary DNAs, wherein said first-strand complementary DNAs are generated by reverse transcription of said messenger RNAs with said first primers;

c. permitting terminal extension of said first-strand complementary DNAs to form a plurality of polynucleotide-tailed first-strand complementary DNAs, wherein said polynucleotide-tailed first-strand complementary DNAs are tailed by multiple copies of deoxynucleotides;

d. incubating denatured said polynucleotide-tailed first-strand complementary DNAs with a plurality of second promoter-containing primers to form a plurality of double-stranded complementary DNAs, wherein said double-stranded complementary DNAs are generated by extension of DNA polymerase activity;

e. permitting transcription of said double-stranded complementary DNAs to form a plurality of amplified messenger RNAs, wherein said amplified messenger RNAs are generated by extension of RNA polymerase activity through the promoter region of said double-stranded complementary DNAs; and

f. contacting said amplified messenger RNAs with said first primer sequences to form a plurality of said polynucleotide-tailed first-strand complementary DNAs, wherein said polynucleotide-tailed first-strand complementary DNAs are generated by reverse transcription of said amplified messenger RNAs.

Defined broadly, the present invention is an RNA amplification method of performing improved messenger RNA enrichment, comprising the steps of:_

a. providing a plurality of messenger RNAs for following steps;_

b. generating a plurality of polynucleotide-tailed complementary DNAs from said messenger RNAs, wherein said polynucleotide-tailed complementary DNAs are reverse-transcribed from said messenger RNAs and tailed by multiple copies of deoxynucleotides;_

c. permitting denatured said polynucleotide-tailed complementary DNAs to form a plurality of double-stranded complementary DNAs, wherein said double-stranded complementary DNA contains a complementary DNA sequence flanked with an RNA polymerase promoter and a polynucleotide-tail; and_

d. incubating said double-stranded complementary DNAs in a plurality of promoter- and primer-dependent extension systems, and thereby providing a plurality of amplified messenger RNAs from said messenger RNAs._

Of course the present invention is not intended to be restricted to any particular form or arrangement, or any specific embodiment disclosed herein, or any specific use, since the same may be modified in various particulars or relations without departing from the spirit or scope of the claimed invention hereinabove shown and described of which the apparatus shown is intended only for illustration and for disclosure of an operative embodiment and

not to shown all of the various forms or modifications in which the present invention might be embodied or operated.

The present invention has been described in considerable detail in order to comply with the patent laws by providing full public disclosure of at least one of its forms. However, such detailed description is not intended in any way to limit the broad features or principles of the present invention, or the scope of patent monopoly to be granted.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 5

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCAGTGAATT GTAATACGAC TCACTATAGG GAATTTTTTT TTTTTTTTTT TTTTTTT 57

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCCCCCCCC CCCCCCCC 18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCATATGGCA TTTAGGTGAC ACTATAGAAG CCCCCCCCCC C 41

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTTTTTTTTT TTTTTTTTTT TTTTTT 26

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCAGTGAATT GTAATACGAC TCACTATAGG GAACCCCCCC CCC 43